Published in final edited form as:

Free Radic Biol Med. 2010 August 15; 49(4): 641–648. doi:10.1016/j.freeradbiomed.2010.05.017.

Transgenic Mice Overexpressing Methionine Sulfoxide Reductase A: Characterization of Embryonic Fibroblasts

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Abstract

Methionine residues in protein can be oxidized by reactive oxygen species to generate methionine sulfoxide. Aerobic organisms have methionine sulfoxide reductases capable of reducing methionine sulfoxide back to methionine. Methionine sulfoxide reductase A acts on the S-epimer of methionine sulfoxide, and it is known that altering its cellular level by genetic ablation or overexpression has notable effects on resistance to oxidative stress and on lifespan in species from microorganisms to animals. In mammals, the enzyme is present both in the cytosol and mitochondria, and this study was undertaken to assess the contribution of each subcellular compartment's reductase activity to resistance against oxidative stresses. Non-transgenic mouse embryonic fibroblasts lack methionine sulfoxide reductase A activity, providing a convenient cell type to determine the effect of expression of the enzyme in each compartment. We created transgenic mice with methionine sulfoxide reductase A targeted to the cytosol, mitochondria, or both and studied embryonic fibroblasts derived from each line. Unexpectedly, none of the transgenic cells gained resistance to a variety of oxidative stresses even though the expressed enzymes were catalytically active when assayed *in vitro*. Noting that activity *in vivo* requires thioredoxin and thioredoxin reductase, we determined the levels of these proteins in the fibroblasts and found that they were very low in both the non-transgenic and transgenic cells. We conclude that overexpression of methionine sulfoxide reductase A did not confer resistance to oxidative stress because the cells lacked other proteins required to constitute a functional methionine sulfoxide reduction system.

Keywords

Methionine sulfoxide reductase A; Mouse embryonic fibroblast; Oxidative stress; Reactive oxygen species; Thioredoxin; Thioredoxin reductase

Methionine and cysteine are the two commonly occurring sulfur-containing amino acids. While the roles of cysteine as an antioxidant and in cell signaling are widely appreciated, only relatively recently has it been recognized that methionine, like cysteine, functions as an antioxidant and as a key component of a system for regulation of cellular metabolism [1]. Efficiency of both methionine and cysteine as antioxidants or as a component of signaling systems depends on their ready interconversion between a reduced form (methionine or cysteine) and an oxidized form (methionine sulfoxide or cysteine disulfide). Virtually all organisms from bacteria to mammals have several methionine sulfoxide reductases (Msr)

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which catalyze the reduction of MetO to Met [2]. Reduction of free or protein-bound methionine sulfoxide allows the methionine to react again with ROS, creating a system with catalytic efficiency in scavenging potentially damaging species. The system also requires thioredoxin and thioredoxin reductase. The net result is the catalytic scavenging of reactive oxygen species by NADPH [See [3] (Fig. 1)].

Oxidation of Met to MetO creates a chiral center, and the S- and R- epimers are substrates for specific methionine sulfoxide reductases. In most organisms the S-epimer is reduced by MsrA and the R-epimer by MsrB, both using thioredoxin as the source of reducing equivalents. MsrA was described well before MsrB so that the majority of the studies in the literature were performed with MsrA. These investigations provide strong evidence supporting the physiological importance of cyclic oxidation and reduction of Met. Knocking out MsrA caused increased susceptibility to oxidative stress in mice [4], yeast [5], and bacteria [6-9]. Conversely, overexpressing MsrA conferred increased resistance in *Drosophila* [9], *Saccharomyces* [10], *Arabidopsis*, [11], PC-12 cells [12], and human T cells [10]. Several studies demonstrated that modulation of MsrA activity altered the level of reactive oxygen species: overexpression lowered the level in PC-12 cells [12] while lens cells lacking MsrA had an increased level [13].

Increased expression of either MsrA in WI-38 cells [14] or MsrB in MOLT-4 leukemia cells [15] also reduced the steady state level of oxidized proteins after a hydrogen peroxide challenge. Accumulation of oxidatively damaged proteins has been hypothesized to be an important mechanism of the aging process [16], and it is notable that overexpression of MsrA in *Drosophila* doubled the lifespan of the flies [9]. To determine whether this remarkable effect on lifespan occurs in mammals, we have created transgenic mice overexpressing MsrA. MsrA is encoded by a single gene but is present both in the cytosol and the mitochondria [17,18]. We created three different transgenic lines in order to alter the MsrA levels in the cytosol, in mitochondria, or in both locations. These mice were used to generate mouse embryonic fibroblasts (MEF) which we have now evaluated for their resistance to oxidative challenges. Since MEF from wild-type mice lack MsrA [19], the transgenic MEF expressing MsrA were expected to exhibit increased resistance to those challenges.

Experimental procedures

Transgenic mice

For transgenic MsrA constructs (GenBank accession number BC014738), cDNA was amplified by polymerase chain reaction (PCR) from a mouse kidney cDNA library (Clontech, Cat# ML5010t). The full coding region of residues 1-233 was used for the WT construct and residues 21-233 for cytosolic targeted construct. The mitochondria targeted construct was made by fusing the sequence encoding the mitochondrial leader peptide of human ornithine transcarbamoylase with that encoding mouse MsrA 21-233. The amplified PCR products were then purified and digested with NcoI and EcoRI before subcloning between the NcoI and the EcoRI restriction sites of the eukaryote expression vector pDrive-CAG (Invivogen) under the control of the cytomegalovirus enhancer and chicken β -actin promoter. The transgenic inserts were released from the cloning vector and microinjected into the pronuclei of fertilized eggs collected from C57BL/6J mice. After culturing in M16 medium overnight, those embryos which had entered the 2-cell stage of development were transplanted into the oviducts of pseudopregnant surrogate mothers. Mice born to these foster mothers were first screened by PCR for the presence of the transgene, and then confirmed by Southern blot for individual transgenic lines. The positive founder mice were bred with wild type C57BL/6J mice to generate the transgenic mice.

Southern blot analysis

Ten micrograms of mouse liver genomic DNA was digested with HindIII and EcoRI, separated by 0.8% agarose gel electrophoresis, and transferred onto nylon membranes (Hybond™-N Amersham). After crosslinking by exposure to ultraviolet light (UV Stratalinker 1800, Stratagene, La Jolla, CA) DNA was hybridized with ³²P-labeled 340-bp Bpu10I fragment of mouse MsrA cDNA. Copy number of transgene was determined from the relative intensity of the hybridizing band to that of the standard band [20] with an FLA-3000 imager (Fujifilm).

Cell culture

MEF were obtained with modifications of a published procedure [21]. A single cell line was established for each genotype and used in all studies reported here. They were obtained from 13.5 day post-coitum embryos and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) and 100 µg/ml gentamicin. MEF from one embryo were grown to confluency in a 15 cm culture dish and frozen in liquid nitrogen at 1.5×10^6 / ml (passage 1). Subsequent passages were obtained after 1:3 splits. Medium was replaced every 3 days unless noted otherwise. Cells used in experiments were at 80-90% confluence and no more than passage 7. MEF were cultured in an atmosphere of 3% O₂ and 5% CO₂ at 37°C. They were counted with a NucleoCounter automated mammalian cell counter (New Brunswick Scientific). Human adult fibroblasts (Coriel AG08470A) were kindly provided by Dr. Gabriella Viteri, National Heart, Lung, and Blood Institute.

Gel electrophoresis and immunoblotting

MEF near 90% confluence were harvested with RIPA lysis buffer (R0278, Sigma) supplemented with a 1:1000 dilution of protease inhibitor cocktail (P8340, Sigma,) and protein concentrations determined by the bicinchoninic acid method (Thermo Scientific, USA). SDS-PAGE of 5µg of total protein was run on 10-20% Tris-glycine gradient gels (Invitrogen, USA) and transferred onto 0.45µM nitrocellulose membrane (LC2001, Invitrogen). After blocking in Odyssey blocking buffer (927-40000, Li-Cor), the membranes were incubated with a 1:25,000 dilution of primary rabbit anti-MsrA (this laboratory) and mouse anti-GAPDH (G8795, Sigma) antibodies overnight at 4°C. The mouse anti-TrxR1 antibody was purchased from Santa Cruz Biotechnology (sc-28321). The rabbit anti-Trx1, anti-Trx2, and anti-TrxR2 antibodies were kindly provided by Dr. Duck-Yeon Lee of the National Heart, Lung, and Blood Institute. The secondary antibody incubation was performed at room temperature for 1 hour with a 1:10,000 dilution of Alexa Fluor 680 goat-anti-rabbit IgG (A21109, Invitrogen) or IRDye 800CW goat-anti-mouse IgG (926-32210, Li-Cor). Quantitation of fluorescence was performed with a Li-Cor Odyssey infrared scanner with 700nm and 800nm channels.

MsrA activity assay

MsrA activity was measured using L-methionine [R,S] sulfoxide (Sigma) as the substrate. The reaction mixture for activity assay contained 10 mM MetO, 10 mM dithiothreitol in 50 mM sodium phosphate buffer at pH 7.4. Cell extract containing 100 µg protein was added to the reaction mixture, incubated at 37°C for 30 min, and the reaction terminated by addition of 1/20th volume of 10% trifluoroacetic acid. The reaction product was analyzed by amino acid analysis as described [3].

Subcellular fractionation

MEF reaching 90% confluency were swollen in ice-cold hypotonic buffer (10 mM Hepes, pH 7.4, 5 mM MgCl₂, 40 mM KCl, 1 mM diethylenetriamine pentaacetic acid, and 1:1,000

dilution of protease inhibitors cocktail (P8340, Sigma) for 60 min, then aspirated 15 times through a 28.5-gauge needle. The homogenate was centrifuged at 600 *g* for 5 min and the "low-spin pellet" was saved for further analysis. The resulting supernatant was removed and further centrifuged at 11,000 *g* for 10 min to generate the "high-spin pellet" containing mitochondria and the soluble "cytosolic fraction" devoid of mitochondria. Low-spin and high-spin pellets were washed 3 times with mitochondria buffer A (E2778, Sigma) and then sonicated briefly on ice. The final volume of each fraction was measured to allow calculation of MsrA in each subcellular compartment. The purity of each fraction was evaluated by Western blot using a mouse monoclonal anti-pig α -tubulin (sc-8035, Santa Cruz) and mouse monoclonal anti-human porin (A31855, Invitrogen) as cytosolic and mitochondrial probes respectively.

Cyanogen bromide cleavage and amino acid analysis

CNBr cleaves peptide bonds at the carboxyl side of Met but does not cleave at MetO [22]. Cleavage was carried out with minor modifications from that published earlier [23]. Duplicate aliquots containing 10 μ g protein from MEF cell lysates were dried by vacuum centrifugation in 0.5 ml glass vials fitted with Teflon-lined caps. To one of the duplicates, 200 μ l of 100 mM CNBr in 70% formic acid was added, and the vial was incubated at 70°C for 1 hour. Following hydrolysis, amino acid analysis of both aliquots was performed using as Apex C₁₈ column (4M15310 Jones Chromatography, Englewood, CO). MetO content is expressed as a percentage of Met + MetO.

WST cell viability and Flow Cytometry assay

Water-soluble tetrazolium (WST) salt cleavage was utilized as the cell viability assay. The cleavage of tetrazolium to formazan by cellular mitochondrial dehydrogenase is directly proportional to the number of living cells. The formazan dye produced was quantified by measuring the absorbance of the dye solution at 440 nm. MEF were seeded in a 96-well plate at 10,000 cells per well. After overnight culture during which the cells attached to the plate, medium was removed and the cells were gently washed with phosphate buffered saline (1.1 mM KH₂PO₄, 3.0 mM Na₂HPO₄, 155 mM NaCl, pH 7.4; Invitrogen 10010). Freshly prepared reagent in DMEM was then added to each well and incubated for the indicated time: hydrogen peroxide (50-200 μ M, 24 hr), paraquat (200-800 μ M, 24 hr), rotenone (0.5-5 μ M, 5 hr) or chromium (10-100 μ M, 5 hr). After treatment, the medium was removed and fresh DMEM with 10% fetal bovine serum was added to each well for another 6 hours. For exposure to glycine chloramine (20-100 μ M), Hanks' balanced salt solution without serum was substituted for DMEM to avoid interaction and exchange with amino acids in DMEM [24]. After 6 hr, the medium was changed back to DMEM with serum. Cell viability was determined with a Quick Cell Proliferation Assay (K302-500, Biovision). Cell viability of treated cells was normalized to that of the untreated cells.

For flow cytometric assays, MEF cultured in 3.5 cm culture dish were treated with 50 μ M glycine chloramine in Hanks' balanced salt solution for 30 min. Cells were then harvested and stained with calcein AM and ethidium homodimer-1 (L3224, Invitrogen). The ratio of live:dead cells was determined in an LSRII flow cytometer (Becton-Dickinson) equipped with lasers emitting at wavelengths 488 and 633 nm under the control of FACSDIVA 6.1.1 software. Data were analyzed with Flowjo software (Version 8.8.6, Tree Star, San Carlos, CA).

All experiments were performed at least twice. The number of replicates and the passage number of the MEF are stated in the legend of each figure. Plots with error bars display the average and standard error of the mean.

Results

Generation of MsrA transgenic mice

We constructed three MsrA transgenic DNA constructs for overexpression of MsrA in the cytosol, the mitochondria, and in both compartments. The last was simply the wild type sequence which encodes full length mouse MsrA with residues 1-233 (Fig. 1A). For the TgMito construct we replaced the MsrA mitochondrial targeting sequence with the strong mitochondrial targeting sequence from human ornithine transcarbamoylase (Fig. 1B) [25]. The cytosolic-targeting construct deleted the mitochondrial targeting sequence and thus contained just the sequence encoding residues 21-233 (Fig. 1C). All 3 constructs led to the generation of germ-line transmitted, viable transgenic mice. Southern blot analysis was utilized to estimate the copy number of inserted transgenes (Fig. 2). Copy number did not correlate well with tissue protein levels (Figs. 2C and 2D), probably due to the effects of the integration sites, increased DNA methylation, or chromatin packaging [26]. The detailed characterization of each transgenic line, including lifespan studies, will be reported in a future publication.

MsrA protein, activity, and subcellular distribution

To provide physiological gas concentrations, MEF were cultured in 3% oxygen and 5% carbon dioxide. The growth rates of the MEF are shown in Fig. 3. Initial growth rates after splitting were similar, although the number of cells at confluence differed among the lines. Western blot and activity assay confirmed our previous observation that non-Tg MEF lack detectable MsrA [19], providing a good control to assess the effect of overexpression of MsrA (Fig. 4A). We also prepared fibroblasts from skin biopsies of adult mice and could not detect MsrA in them nor in fibroblasts from adult humans¹ (not shown). However, we readily detected MsrA in the 3 transgenic MEF. Taking the TgMito expression level as 1, that of the TgCyto is 4 and that of the TgWt is 8. The enzymatic activities correlated well with the protein levels (Fig. 4B).

Our goal was to target MsrA either to the cytosol, to mitochondria, or to both. Fig. 5 shows that this occurred. The ornithine transcarbamoylase targeting sequence in the TgMito directed virtually all of the MsrA to the mitochondria. The TgCyto construct lacks a targeting sequence and thus all of the MsrA was cytosolic. The TgWt had ~40% mitochondrial and ~60% cytosolic localization.

MsrA overexpression does not lower MetO content nor protect MEF against oxidative stresses

Mouse tissues typically have about 5% of their methionine residues in the oxidized form, except for brain which is about 8% [29]. We found the levels in non-transgenic MEF were much higher at 15%. Overexpression of MsrA did not affect the MetO content (Fig. 6).

Since non-transgenic fibroblasts lack MsrA and overexpression of MsrA has previously been shown to protect cardiac myocytes as well as vascular smooth muscle from oxidative stress [30,31], we expected to observe similar protection for the transgenic fibroblasts. Hydrogen peroxide can directly oxidize biological molecules and participate in Fenton chemistry, paraquat redox cycles and generates superoxide [32-34], rotenone interrupts mitochondrial electron transport at complex I [35], and chromium generates ROS and causes oxidative damage to proteins which was mitigated in yeast by overexpression of MsrA [36,37]. We observed no protection of MEF against any of these agents (Fig. 7). Glycine

¹However, the human WI-38 cell line does express MsrA [27]. The line was derived from human fetal lung and has fibroblast-like characteristics [28].

chloramine is cell permeable and preferentially reacts with thiols and methionine residues [38]. Despite this relative specificity, overexpression of MsrA did not protect MEF from chloramines toxicity (Fig. 8).

Thioredoxin and thioredoxin reductase levels in MEF

The inability of overexpressed MsrA to lower protein MetO content and to protect MEF from oxidative stress was unexpected. *In vivo*, MsrA's ability to reduce MetO requires thioredoxin and thioredoxin reductase. If either of these co-members of the methionine-methionine sulfoxide redox cycle were deficient then MsrA would not be functional even if overexpressed. We therefore determined the levels of thioredoxin and thioredoxin reductase in MEF and compared these to liver, a tissue known to have the complete methionine sulfoxide reduction system. Thioredoxin-1 (Trx1) and its reductase (TrxR1) are found in the cytosol while thioredoxin-2 (Trx2) and its reductase (TrxR2) are mitochondrial. We found that the levels of Trx1 were comparable in liver and MEF, but its reductase was markedly decreased in MEF (Fig. 9). The mitochondrial Trx2 and TrxR2 were both quite low compared to liver (Fig. 10). Thus, neither the cytosol nor the mitochondria of MEF have a complete enzymatic system required to support the activity of MsrA.

Discussion

As outlined in the introduction, there exists substantial experimental evidence that the level or activity of MsrA affects the resistance of a variety of cells and organisms to oxidative stress. Having found that mouse and human fibroblasts do not have detectable MsrA, we expected that expression of the enzyme would bolster their resistance to oxidative challenges. This was not the case, even for challenge with a chloramine which preferentially oxidizes sulfur containing amino acids. We considered and investigated several possible explanations for this unexpected result. We confirmed that the protein is expressed and is catalytically active in the MEF derived from the transgenic mice. MsrA is itself oxidized when it reduces a molecule of MetO and its active form must be regenerated by a system which includes thioredoxin and thioredoxin reductase. Different isoforms of each of these proteins are directed to mammalian cytosol and mitochondria. While expression of MsrA did not alter the levels of these proteins, we found that both the cytosolic and mitochondrial forms of thioredoxin reductase are present at very low levels, if at all (Figs. 9 and 10). Thus, overexpression of MsrA cannot augment the system for reduction of MetO.

MEF from non-transgenic as well as from MsrA overexpressers are functionally knockouts for MsrA activity. Consistent with this phenotype, we observed that ~15% of methionine residues in MEF proteins have been oxidized to the sulfoxide. This can be compared to a typical *in vivo* tissue content of 5% [29]. A high content of oxidatively modified proteins is likely to increase the rate of turnover of proteins and also apoptotic death [39,40]. When these proteins turn over *in vivo*, the released MetO is presumably released to nearby cell types which do have MsrA [41] or transported to organs rich in MsrA such as the liver and kidney where it is reduced back to Met.

Parrinello and colleagues showed that oxygen sensitivity limits the replicative lifespan of mouse fibroblasts grown in 20% oxygen (room air) and suggested that the limitation is due to oxidative DNA damage [42]. Meshing well with earlier reports from a number of laboratories [43], they found that MEF grown in 3% oxygen rather than room air grew faster, reached higher saturation densities, and did not senesce. The oxygen concentration experienced by fibroblasts *in vivo* likely differs widely from organ to organ and even within an organ, with 3% oxygen or less being well within the physiologically normal concentration [44]. It will be interesting to examine the phenotype and resistance to oxidative stress of MEF in which the system for repair of MetO is made fully functional.

Acknowledgments

We thank Angelique Biancotto and J. Philip McCoy of the Flow Cytometry Core Facility of the National Heart, Lung and Blood Institute for their assistance with the flow cytometry. This research was supported by the Intramural Research Program of the National Heart, Lung, and Blood Institute.

Abbreviations

Cyto	cytosolic
DMEM	Dulbecco's modified Eagle's medium
MetO	methionine sulfoxide
Msr	methionine sulfoxide reductase
MEF	mouse embryonic fibroblasts
Mito	mitochondrial
MTS	mitochondrial targeting sequence
ROS	reactive oxygen species
Tg	transgenic
TgCyto	Transgenic targeted to the cytosol
TgMito	Transgenic targeted to the mitochondria
TgWt	Transgenic targeted to both the cytosol and mitochondria
Trx	thioredoxin
TrxR	thioredoxin reductase
WST	water-soluble tetrazolium.

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A) **TgWt** : B) **TgMito** : C) **TgCyto** : **Figure 1.**

The scheme illustrates the differences among transgenic MsrA targeting constructs. A) TgWt. Full length (1-233) mouse MsrA protein for TgWt transgenic mice. The endogenous mitochondrial targeting signal (MTS, residues 1-20, black bar) is intact. Differential initiation at Met-1 or Met-21 targets MsrA to both the cytosol and mitochondria [45]. B) TgMito. The mouse MTS was replaced with the strong MTS from human ornithine transcarbamoylase (hatched bar). C) TgCyto. The MTS was deleted, thus targeting MsrA only to the cytosol.

Figure 2. Southern blot for MsrA. Genomic DNA was obtained from liver and 10 µg was loaded in each lane. A) Lanes 1-4 are from TgMito, TgWt, non-Tg, and TgCyto mice respectively. The numbers at the right of the panel indicate the size of 4.5 kb endogenous and 3.1kb transgenic MsrA bands. B) Copy number of transgenic MsrA (gray bar) and endogenous MsrA (black bar), calculated as described in Experimental Procedures. C) Western blot of liver homogenate containing 10 µg total protein. D) Quantitation of the MsrA protein content from panel C. The values shown for the Western blot and for activity are the mean and SEM from 3 animals.

Figure 3.

Growth of MEF. Duplicate plates of each line were seeded with 50,000 cells at passage number 3 and grown to confluence. The media was changed every other day. The plot displays the mean cell number with its SEM. The experiment was repeated twice.

Figure 4. MsrA protein level and enzymatic activity in MEF. A) Western blot of MsrA levels in MEF. A total of 5 μ g of protein was loaded on each lane. MsrA was not detectable in non-Tg MEF. GAPDH served as a loading control. B) Quantitation of MsrA protein levels from panel A. C) MsrA enzymatic activity. The values shown are the average and SEM from 2 separate experiments.

Figure 5.

Subcellular distribution of MsrA in MEF. A) Western blot for MsrA in cytosol and mitochondria. Markers were detected in the 800 nm channel while MsrA was detected in the 700 nm channel. The amount of protein loaded on the gels was adjusted to allow accurate quantitation so that the intensities for the two fractions cannot be compared directly. B) Percentage of total MsrA in the mitochondria. These were calculated by taking into account the differences in volume of the cytosolic and mitochondrial fractions as well as the fraction loaded in panel A. The results are the average of two separate experiments.

Figure 6. MetO as a fraction of total Met and MetO content in MEF proteins. The MEF were at passage number 5. The values shown are the mean and SEM from 3 separate analyses.

Figure 7.

MEF derived from transgenic mice are as susceptible to oxidative stress as MEF from non-transgenic mice. Cells were treated with paraquat, chromium, hydrogen peroxide, or rotenone. Viability was calculated by comparison to untreated controls. The MEF were at passage number 4. The bars display the average and SEM from two separate experiments.

Figure 8.

MEF derived from transgenic mice are equally sensitive to glycine-chloramine. A) WST assay of viability of MEF treated with 0-100 μ M glycine chloramine; B) Flow cytometric analysis of viability of MEF treated with 50 μ M glycine chloramine. The assays were repeated in two separate experiments from passage numbers 4 and 5.

Figure 9.

Comparison of cytosolic Trx1 and TrxR1 levels in MEF and liver. 10 μ g protein from MEF or liver was loaded in each lane. A) Relative level of TrxR1 in MEF and liver; B) Relative level of Trx1 in MEF and liver; C) Western blots of Trx1 and TrxR1 in MEF and liver demonstrating the marked difference in content between MEF and liver. GAPDH was used as a loading control. The MEF were at passage numbers 5-7. The results were obtained from two separate experiments.

Figure 10.

Comparison of mitochondrial Trx2 and TrxR2 levels in MEF and liver. 10 μ g protein from MEF or liver was loaded in each lane. A) Relative level of TrxR2 in MEF and liver; B) Relative level of Trx2 in MEF and liver; C) Western blots of Trx2 and TrxR2 in MEF and liver, demonstrating the marked difference in content between MEF and liver. GAPDH was used as a loading control. The MEF were at passage numbers 5-7. The results were obtained from two separate experiments.